Comparison of Sample Preparation Procedures for Colorimetric Analysis of Nitrite in Frankfurters

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Three groups of methods for analyzing nitrite in meat are compared. All methods consist of a sequence of steps, grouped according to initial extraction procedures. In Group 1, the sample is treated with borate followed by $HgCl_2$ or Carrez I, and then Carrez II. In Group 2, the sample is diluted with water, heated at 80°C, and analyzed immediately (AOAC) or after addition of either Na_2CO_3 and $FeCl_3$ or $HgCl_2$. In Group 3, the sample is made alkaline with NH_4Cl buffer and then treated with one of the following: activated carbon plus Carrez I and II, alumina cream, or $AlK(SO_4)_2$. At each step when the method involved the addition of a chemical, supernates and precipitates (if formed) were analyzed for nitrite by Griess reagent both before and after AOAC digestion. The normally discarded precipitates formed after addition of $HgCl_2$ and Carrez I and II contained bound nitrite that could be detected by AOAC analysis. Except in the AOAC method, $HgCl_2$ improved nitrite analysis. Results by AOAC analysis were 3 to 300% higher than those determined after addition of any chemical or combination of chemicals. Spiked meat samples could not be used in comparing nitrite analysis methods because results were misleading. Acid meat samples, such as fermented sausages, required neutralization before AOAC analysis.

Interest in nitrite levels in meat has heightened in recent years because of its possible connection with low concentrations of nitrosamines discovered in some cured meat products, particularly fried bacon. Efforts to reduce the concentration of nitrite in these products require accurate and reproducible analytical procedures. Standard methods have been adopted by different organizations in various parts of the world. The method approved by the AOAC has been accepted as the standard in the United States. Other organizations, such as the International Organization for Standardization (ISO), have established standardized methods, and other investigators have used a number of other methods which frequently appear in the literature.

We compared 12 of these procedures and found differences in the concentration of nitrite determined. Although all of the methods determine nitrite by the diazotization of an aromatic amine followed by coupling with an amino or hydroxy aromatic compound to form a colored azo dye, they vary considerably in procedures for preparing samples for analysis. A number of procedures, including those of Mirna (1), Grau and Mirna (2), and the ISO (3), use borate to extract the nitrite. A second group, involving the methods of the AOAC (4), the Norwegian Food Committee (5), and Acél (6), use heat to deproteinize and clarify sample solutions. The methods described by Adriaanse and Robbers (7), Coppola *et al.* (8), Kamm *et al.* (9), Follett and Ratcliff (10), and Glover and Johnson (11) specify addition of alkali to extract nitrite. We shall refer to these 3 groups as the "borate," "heated," and "alkaline group," respectively. To a certain extent, the reasons for most of the reagents or the procedures for preparing meat extracts for nitrite analysis are obscure. Iron (Carrez I, $FeCl_3$), Zn (Carrez II), Hg, $B_4O_7^{2-}$, and Al are protein precipitants, but it has not been established that the turbidities that develop during sample preparation are in fact proteinaceous. Although nitrosothiols undergo facile hydrolysis in the presence of mercury (12), there is no direct evidence that Hg as used in the various procedures does indeed cleave them. Alkali is used to neutralize or alkalize meat extracts to prevent acid-catalyzed oxidation of nitrite, but the efficacy of the procedure has not been demonstrated. Heating, as

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Table 1. Concentrations of reagents used in the original 12 procedures and concentrations used in this study

Compound	Literature[a]		This study	
	Reagent concn, *M*	Working concn, *mM*	Reagent concn, *M*	Working concn, *mM*
Carrez I	0.25 (3), 0.36 (8)	4.2 (3), 6.5 (8)	0.3	5.8
Carrez II	1.04 (1,2,8), 0.76 (3)	31 (1), 6.3 (2), 12.8 (3), 18.9 (8)	1.0	19.2
$Na_2B_4O_7$	sat. (0.13) (1,2,3)	21.7 (1), 39.4 (2,3)	sat. (0.13)	39.4
$HgCl_2$	sat. (0.18) (1)	13.8 (1)	sat. (0.18)	16.4
Na_2CO_3	0.57 (5), 2.34 (7)	49.6 (5), ~8.4 (7)	0.5	50
$FeCl_3$	0.5 (5)	21.7 (5)	0.5	25
NH_4Cl	0.67 (8), 0.24 (9,10,11,12)	31.9 (8), 6 (9), 18.5 (10), 14.1 (11), 12.6 (12)	0.5	16.7
$AlK(SO_4)_2$	sat. (0.24) (9,11,12)	~4.8 (9)	sat. (0.24)	15.7

[a] Reference citations are given in parentheses.

in the AOAC procedure, presumably denatures proteins and cleaves nitroso groups. What is perhaps the greatest shortcoming is that the effectiveness of these various procedures in relation to each other has never been assessed. We undertook such a comparison to determine the most effective procedure, to establish the effects of the procedures on each other, and to gain insight as to just what the various procedures were doing to the cured meat extracts.

Experimental

Frankfurters were used as the cured meat product for the nitrite analyses. The emulsion was prepared in our laboratories by a standard formula and processing schedule (unpublished information). Nitrite was added to give an initial concentration of 125 ppm. Other reagents added are given in Table 1. The data in Tables 2, 3, and 5 are for frankfurters made from the same batch of emulsion. The data in Table 4 are for another batch, and the data in Table 6 are for a package of commercial frankfurters. Whole slurries are analyzed in all the procedures studied. We used both whole frankfurter-water slurries and slurries separated by centrifugation into soluble and insoluble fractions. The effect of each treatment was compared with the value obtained by a standard treatment which consisted of the AOAC procedure on a whole slurry.

We had 2 choices for performing the multiple comparisons: either proceed directly as the original authors proposed, or prepare a composite procedure for each group. Since our intent was to determine the relative effectiveness and interactions of each step, we decided to perform stepwise procedures on the same sample extract. This required using a composite procedure. This, in turn, required some compromises in reagent concentrations and techniques, but for the most part, such compromises were minor. Reagents used in the experiments are shown in Table 1. The concentrations described in the original procedures are given, as well as the concentrations we selected as a compromise to the various levels. The only major deviation was the use of 15.7*mM* $AlK(SO_4)_2$. This concentration was required to adjust the pH of the alkaline sample to 6.0, which Follett and Ratcliff found was necessary for subsequent colorimetric analysis in acid solution.

The flow diagrams for sample preparation by the borate and alkaline group treatments are shown in Figs 1 and 2, respectively. One procedural compromise was made. Extraction with hot water or heating during sample preparation is part of the procedures of Grau and Mirna (2) (borate group) and Coppola *et al.* (8), Follett and Ratcliff (10), and Glover and Johnson (11) (alkaline group). Since we heated the extraction solutions at each step of chemical addition, the net effect of heating is effectively covered in the "after AOAC digestion" data in the tables. Figure 1 shows details of the treatments involved in the borate group. The frankfurter sample, with 2 parts water added, was reduced to a slurry in a blender. Borate ($Na_2B_4O_7$) and $HgCl_2$ were added to a portion of the slurry. This mixture was centrifuged, and Carrez II (potassium ferrocyanide) was added to the supernate. The precipitate was taken up in 2 volumes of water and Carrez II was added. Another portion of the original meat slurry was centrifuged, and Carrez I (zinc acetate) and Carrez II were added to the supernate. The precipitate was dispersed in water, and Carrez I and II were added.

In the procedure for the heated group, the frankfurter sample was once again made into a slurry, and a portion was diluted 100 times with water and heated 2 hr at 80°C. Na_2CO_3 and $FeCl_3$ were then added either singly or in combination. The same reagents were added to an-

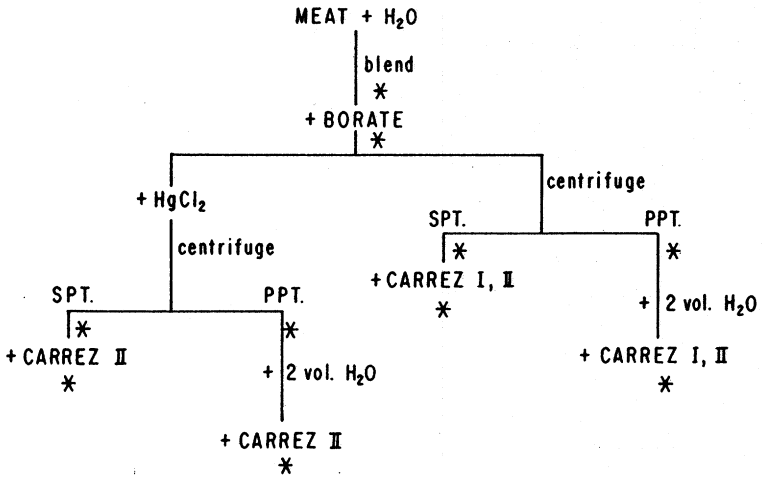

FIG. 1—Borate group treatment for sample preparation. The asterisk indicates samples taken for Griess nitrite analysis before and after AOAC digestion of sample. For whole slurry analyses (Table 2), samples were not centrifuged. Preparative procedure was same as that for supernate (spt) alone.

other portion of frankfurter slurry, followed by the same heat treatment.

Figure 2 shows the procedure for the alkaline group. The frankfurter slurry was made alkaline with NH_4Cl or NH_4OH. Water and charcoal were added to a portion of the alkaline slurry, and sample was shaken 0.5 hr. Another portion the alkaline slurry was centrifuged. The supernate was divided; and one portion was treated with charcoal. The remainder of the supernate was again divided, and Carrez I and II were added to one half, $AlK(SO_4)_2$ to the other half. The precipitate was dispersed in water, and Carrez I and II were added to one half, $AlK(SO_4)_2$ to the other half.

After each step of the above procedures, the fraction was analyzed for nitrite both before and after digestion, which consisted of diluting the sample 1:100 with water and heating 2 hr at 80°C, according to the AOAC method (4).

The concentration of nitrite was determined in all samples with Griess reagent, which is a mix-

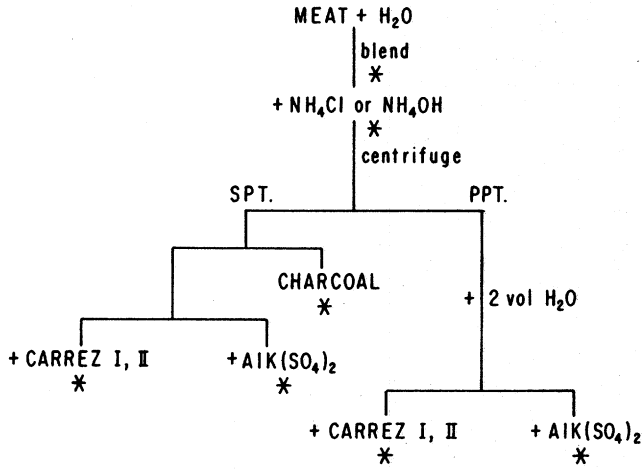

FIG. 2—Alkaline group treatment for sample preparation. The asterisk indicates samples taken for Griess nitrite analysis before and after AOAC digestion of sample. For whole slurry analyses (Table 5), samples were not centrifuged. Preparative procedure was same as that for supernate (spt) alone.

Table 2. Sodium nitrite values (ppm) from borate group procedure on a noncentrifuged frankfurter-water slurry

Treatment	Before AOAC dig.	After AOAC dig.
None	11 T[a]	19
Borate	9	18
Borate + $HgCl_2$	16 T	18 T
Borate + $HgCl_2$ + Carrez II	14 T	17 T
Carrez I + II	9 T	14

[a] T indicates a turbid solution.

ture of 8.17*mM* sulfanilic acid and 2.18*mM* 1-naphthylamine[1] in 15% acetic acid. Nitrite and sulfanilic acid form a diazonium salt which couples with 1-naphthylamine to produce a red azo dye with an absorption maximum at 525 nm. All nitrite concentrations shown are calculated back to the original sample.

In a number of instances, the reagents used produced turbidity in the azo dye solutions. Light beam attenuation due to turbidity is approximately the same throughout the visible range, but the Griess pigment absorption at 600 nm is only 1–2% that at 540 nm. If the 600 nm absorption was below 0.040 AU, we made an approximate correction of the nitrite values by subtracting the absorbance at 600 nm from the 540 nm absorbance. However, absorption is intensified by turbidity (13) and we found that if the absorbance was above 0.040 A.V. at 600 nm, the turbidity was too great to apply such corrections.

Results and Discussion

The effect on nitrite analysis of using the borate sample preparation on a noncentrifuged frankfurter-water slurry is shown in Table 2. All the nondigested samples were turbid. After the heat digestion treatment, however, the samples to which nothing or Carrez I and II had been added were clear. The sample receiving no treatment had a higher nitrite value than the sample treated with Carrez I and II. We had investigated the use of borate alone in previous studies (unpublished data) and found it did not significantly influence nitrite values.

The results of borate treatment of a centrifuged frankfurter-water slurry are shown in Table 3. The supernates before digestion, which are the solutions usually analyzed for nitrite content, were still turbid. The higher nitrite values after digestion of the precipitates resulting from addition of borate-$HgCl_2$ with and without Carrez II indicate that nitrite is lost in the precipitate when these reagents are used. This is an important point because these precipitates are discarded in other methods. When no treatment is used after digestion, the whole sample value is the same as the supernatant value but less than the precipitate value, suggesting that either some compound in the supernate is interfering in the analysis or that nitrite is loosely bound in the precipitate.

The results of the heated series are shown in Table 4. Heating at 80°C for 2 hr gave a clear solution. The turbidity in the samples from the treatment in which Na_2CO_3 or $FeCl_3$ was added and then heat applied was so great that they could not be read accurately in a spectrophotometer. Addition of Na_2CO_3 and $FeCl_3$ and then heat gave no improvement over heat alone. Heat followed by addition of Na_2CO_3 gave an apparent improvement but also caused turbidity. Heat followed by $FeCl_3$ caused considerable turbidity. Heat followed by addition of Na_2CO_3 and $FeCl_3$ offered no improvement over heat alone.

The alkaline group values for a noncentrifuged slurry are shown in Table 5. Addition of NH_4Cl-charcoal and NH_4OH-charcoal gave clear solutions before digestion but the nitrite values were not significantly different from the value after digestion of the sample receiving no treatment.

The effects of the alkaline procedure on nitrite analysis in a commercial frankfurter slurry are shown in Table 6. Commercial frankfurters were used for this procedure because we did not

Table 3. Sodium nitrite values (ppm) from borate group procedure on a centrifuged frankfurter slurry

	Before dig.		After dig.		
Treatment	SPT[a]	PPT[b]	SPT	PPT	Whole sample
None	15 T[c]	10	23	28	22
Borate + $HgCl_2$	16 T	15	20	44 T	
Borate + $HgCl_2$ + Carrez II	18 T	16 T	17	43	
Carrez I + II	16 T	12 T	17	15	

[a] SPT = supernate.
[b] PPT = precipitate.
[c] T indicates a turbid solution.

[1] The compound 1-naphthylamine is listed as a carcinogen under Title 29, Chapter XVII, Occupational Safety and Health Administration, Department of Labor. Necessary precautions for using a less than 1% solution of this compound were taken.

have enough laboratory-made frankfurters to complete the experiment. The treatments improved the values for both supernates and precipitates before digestion and for supernates after digestion. As shown in Table 3, the precipitates after digestion showed higher values than the supernates. The results were confirmed by repeating the procedure with other brands of commercial frankfurter and Lebanon bologna.

An analysis of variance (14) was made of the before- and after-digestion nitrite values in Tables 2, 3, 5, and 6. The F ratio for the chemical treatments was 0.49 (not significant), but the F ratio for before and after digestion was 10.78, significantly different at >99% probability. Because of the relatively large spread of the values after digestion, we made a difference analysis of the before- and after-digestion data groups (14). The Student's t value was 5.54, which corresponds to >99.9% probability that the 2 sets of data are different. Since the values for the untreated samples before digestion appeared to be low, we made an outlier analysis of the before-digestion group (15). The results indicated that all the values were within the expected range of values for one distribution. The conclusion, therefore, is that none of chemical additives had any significant effect on the measured nitrite values. Yet even if any of the chemical treatments had been significantly different, that difference was relatively small. In contrast, the AOAC method applied to samples after the chemical treatments resulted in not only significantly, but appreciably higher yields of measured nitrite. The average of the before-digestion values of Tables 2, 3, and 5 was 14.2±2.4 ppm; the average of the after-digestion values was 22.7±7.8 ppm. We have observed up to an 8-fold increase in measured nitrite as a result of AOAC digestion in duplicate experiments (compare Table 7). In terms of maximal yield of nitrite from meat samples, the AOAC method is clearly superior to the other treatments.

Table 4. Sodium nitrite values (ppm) from heated group procedure on a noncentrifuged frankfurter slurry

Treatment	Concn
2 hr at 80°C	9
Na_2CO_3; 2 hr at 80°C	VT[a]
$FeCl_3$; 2 hr at 80°C	VT
Na_2CO_3 + $FeCl_3$; 2 hr at 80°C	7
2 hr at 80°C; Na_2CO_3	14 T[b]
2 hr at 80°C; $FeCl_3$	VT
2 hr at 80°C; Na_2CO_3 + $FeCl_3$	8

[a] VT indicates a very turbid solution.
[b] T indicates a turbid solution.

Table 5. Sodium nitrite values (ppm) from alkaline group procedure on a noncentrifuged frankfurter slurry

Treatment	Before dig.	After dig.
None	12 T[a]	18
NH_4Cl	14 T	23 T
NH_4Cl + charcoal	16	24
NH_4Cl + $AlK(SO_4)_2$	15 T	24 T
NH_4Cl + Carrez I + II	14 T	24 T
NH_4OH	13 T	26 T
NH_4OH + charcoal	18	22 T
NH_4OH + $AlK(SO_4)_2$	13 T	21 T
NH_4OH + Carrez I + II	14 T	19 T

[a] T indicates a turbid solution.

The compounds we examined, $HgCl_2$, Carrez I and II, $B_4O_7^{2-}$, Fe^{3+}, and $AlK(SO_4)_2$, have been traditionally considered deproteinizing agents, but there is some question as to whether cured meat extracts contain any soluble proteins. The sarcoplasmic proteins of muscle tissue contain tyrosine and tryptophane, both of which absorb at 260 nm. We measured the ultraviolet absorption of the frankfurter extracts of this study as well as other cured meat products—ham, Lebanon bologna, and bacon—and found no 260 nm absorption bands in any. It is thus possible that our null results with respect to the addition of these reagents was due to the absence of soluble protein in our extracts, a condition that might not prevail in other products. The precipitates contained analyzable nitrite which is presumed to have been bound to the insoluble and denatured proteins therein. If part or all of the nitrite in the precipitates had been in the form of nitrosothiols, the addition of mercury would have been expected to result in higher yields of measured nitrite. There was an increase (Table 3) in the measured nitrite in the precipitates with added mercury, but again it was not of the magnitude obtained after AOAC digestion.

Researchers who publish evaluations of analytical methods for nitrite commonly add known amounts of nitrite to meat and judge the method by the per cent recovery. We found this to be an unacceptable and unrealistic technique for evaluating a method for 2 reasons.

Table 6. Sodium nitrite values (ppm) from alkaline group procedure on a centrifuged commercial frankfurter slurry

Treatment	Before dig.			After dig.		
	SPT[a]	PPT[b]	Whole sample	SPT	PPT	Whole sample
None	21 T[c]	26		30	75	45
NH_4Cl	25	33 T		39	70 T	48 T
NH_4Cl + charcoal	39		39	46		49
$NH_4Cl + AlK(SO_4)_2$	29	33		40	77	
NH_4Cl + Carrez I + II	28	31		40	64	

[a] SPT = supernate.
[b] PPT = precipitate.
[c] T indicates a turbid solution.

First, nitrite reacts with meat components such as heme pigments and sulfhydryl groups of proteins and with meat additives such as ascorbate and erythorbate and therefore is not readily available for nitrite analysis. When nitrite is added immediately before analysis, it is likely that the method measures only free nitrite, not those reacted forms which occur in meat in which nitrite has been present for a longer time. Second, lower levels of residual nitrite are found more commonly in commercially cured meat products than the higher levels obtained from the intentional spiking of meat. When meat was spiked with higher levels of nitrite, we found that all methods performed equally well. They did not compare so favorably when meat containing normally low residual levels was used.

An example of nitrite differences between spiked and unspiked meat samples is shown in Table 7. Lebanon bologna, which had been made with nitrite in this laboratory, was analyzed for nitrite both before and after addition of 138 ppm $NaNO_2$. Although three of the analyzed nitrite values are high, they are in the range of 1σ and are therefore statistically not significantly different (16, 17). The spiked samples are all high and, with these data, indicate that mercuric chloride addition was deleterious, which is not true. In contrast, the unspiked samples show that the AOAC digestion resulted in the release of nitrite, even though the amount was small. We conclude therefore that the only valid way to test an analytical nitrite procedure is to use samples in which nitrite has had an opportunity to react with tissue components.

Table 7. Sodium nitrite values (ppm) from 2 treatments of nitrite-spiked and unspiked Lebanon bologna

Treatment	Before dig.		After dig.	
	Spiked[a]	Unsp.	Spiked	Unsp.
None	156	2	168	16
$HgCl_2$	144	3	148	15

[a] Spiked with 138 ppm $NaNO_2$.

Summary

In our examination of techniques for treating meat samples before nitrite analysis, we found that none of them was any improvement over the AOAC digestion method. The combination of borate, $HgCl_2$, and Carrez II appeare precipitate bound nitrite which was released by AOAC digestion. In slurries, borate and $HgCl_2$ resulted in an increase in measured nitrite, yet even then AOAC digestion resulted in an increased yield. The remaining treatments, Carrez I, Na_2CO_3, $FeCl_3$, NH_4Cl, and $AlK(SO_4)_2$ offered no improvement or caused considerable turbidity. Although all of these chemicals are commonly used in nitrite analysis in meat, we would not recommend their addition. The AOAC method, although not without drawbacks, still appears to be a better and simpler method when tested on residual nitrite in cured meats than the 11 others we examined.

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